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Physiological and Morphological Identification of Photosensitive Neurons in the Opisthosomal Ganglia of *Limulus polyphemus*

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Abstract. The motor outputs of the isolated opisthosomal ventral nerve cord in *Limulus polyphemus* are modulated by light. We have identified the photosensitive neurons and examined their physiological and morphological properties using intracellular recording and staining techniques. We found that photosensitive neurons are present in each ganglion of the opisthosomal ventral nerve cord. These neurons often discharged action potentials spontaneously in the dark, and they increased the frequency of this discharge in the light. The mean latency (±SD) of the light-induced action potential was 2.2 ± 1.1 s. Cells responded in a graded fashion over a 2-log unit of light intensity. The peak spectral sensitivity was 425 nm or lower. The Lucifer-yellow-labeled photosensitive neurons had oval somata with mean (±SD) diameters of 102 ± 3 μm (long axis) and 75 ± 5 μm (short axis), and extended their axons to the contralateral region of the ventral nerve cord. The soma had no dendrites, and the axon had thin branches.

Introduction

Extra-retinal photoreceptors have been detected in the central nervous system of arthropods (for review, see Page, 1982). For example, photosensitive neurons have been found in the 6th abdominal ganglion of crayfish (Prosser, 1934; Kennedy, 1958, 1963; Bruno and Kennedy, 1962), in the abdominal ganglion and the brain of insects (Arikawa *et al.*, 1991; Ichikawa, 1991; Hariyama, 2000), in the brain of spiders (Yamashita and Tateda, 1981, 1983), and in the telson (Zwicky, 1968) and the metasomata of scorpions (Geethabali and Rao, 1973). Among these extra-retinal photoreceptors, the caudal photoreceptors in crayfish have been studied extensively because they are accessible for intracellular recording (Wilkens and Larimer, 1972, 1976; Kruszewska and Larimar, 1993) and because their activity can be correlated with the animal's behavior (Welsh, 1934; Edwards, 1984; Simon and Edwards, 1990).

The horseshoe crab, often referred to as a "living fossil," has proved useful for studying the phylogenetic development of arthropods. Because of its accessible eyes, the animal is also an admirable model for vision research: extensive studies have been performed on its lateral eyes (Hartline and Ratliff, 1957; Ratliff, 1974; Barlow *et al.*, 2001) and ventral photoreceptor (for review, see Dorlöchter and Stieve, 1997). Studies on extra-retinal photoreceptors in the central nervous system have, however, been limited (Snodderly, 1971).

Using extracellular recording techniques, we recently observed that motor outputs of the isolated opisthosomal ventral nerve cord of *Limulus* are modulated by direct light stimulation (Mori and Kuramoto, 2004). Encouraged by this finding, we used intracellular recording techniques to search for photosensitive neurons in isolated ganglia of opisthosomal ventral nerve cord and examined their physiological and morphological properties. The results indicate that photosensitive neurons are present in each ganglion of opisthosomal ventral nerve cord, and the peak of their spectral response is 425 nm or lower. The functional significance of the photosensitive neurons in the *Limulus* central nervous system is compared with those found in other arthropods.

Materials and Methods

Preparation

The horseshoe crabs (*Limulus polyphemus* (Linnaeus, 1758), both sexes, 17–21 cm in carapace width) were purchased from the Marine Resource Center (Marine Biological Laboratory, Woods Hole, MA). They were reared in the

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aquaria of Shimoda Marine Research Center (Shizuoka, Japan) under a natural light/dark cycle. The temperature in the aquaria ranged between 11 and 25 °C throughout the year.

For these experiments, an animal was inverted, and the gill plates together with the opisthosomal ventral nerve cord were dissected free from the animal. Under a stereomicroscope, a chain of 9th to 16th opisthosomal ganglia with intact dorsal and ventral nerves were freed from surrounding tissue. They were put into a petri dish filled with physiological saline solution and kept at 4 °C until used. In every experiment, a single ganglion was isolated from a chain of opisthosomal ganglia and mounted ventral side up on a silpot platform in an acrylic chamber ($3 \times 2 \times 0.7$ cm^3). The sheath of the ganglion and the connective tissue surrounding cell bodies were carefully removed. The preparation was perfused with saline solution saturated with air by bubbling. The saline solution contained (in m*M*) 530 NaCl, 10.7 KCl, 18.0 $CaCl_2$, 24.6 $MgCl_2$, 2.3 $NaHCO_3$, and 3 glucose; adjusted to pH 7.4. The temperature of the saline was maintained between 17 and 19 °C during the experiment.

Electrodes

For intracellular recordings from photosensitive neurons, micropipettes were made from glass capillary tubing (BF100-86-10, Sutter) using a Brown-Flaming micropipette puller (P-97, Sutter) and filled with 3 *M* KCl solution. The resistance of the electrodes was 20–30 MΩ. The electrode was connected to a DC amplifier (MEZ-8301, Nihon Kohden) through a high-impedance negative-capacitance preamplifier (JZ-101J, Nihon Kohden). For extracellular recording of segmental nerves, glass suction pipettes filled with saline were connected to an AC amplifier (AB621G, Nihon Kohden). For intracellular staining, the micropipettes were filled with 3% Lucifer yellow (Polyscience Inc.) in 100 m*M* LiCl (15–80 MΩ). All nerve signals were digitized (Digidata 1200B, Axon Inst.), saved on a computer, and analyzed with Axoscope software (version 9, Axon Instruments).

Light stimulus

The preparation was illuminated with a halogen lamp (300 W) through a heat-absorbing filter, lens, mechanical shutter, and mirror system. The intensity of light of 0.0 log units roughly corresponds to 1.5×10^{17} quanta/cm^2·s at the surface of the preparation and is attenuated with neutral-density filters between 0.0 and −3.0 log units. Monochromatic light (425, 450, 475, 500, 550, and 600 nm) was obtained by narrow-band interference filters (Optical Coatings Japan) in the light path. Neutral-density filters adjusted the quantal flux for each filter to 1.6×10^{14} quanta/cm^2·s.

Intracellular staining

Lucifer yellow was iontophoretically injected into physiologically examined cells with negative electrical currents of 5–15 nA at 2.5 Hz for 30–90 min. Ganglia containing stained cells were kept overnight at 4 °C and then fixed for 2 h in 4% paraformaldehyde in 0.1 mol/l phosphate buffered saline (PBS) at pH 7.4. After washing for 1.5 h in PBS and several minutes in distilled water, they were dehydrated in a graded ethanol series and cleared in methylsalicylate. The stained neurons in whole-mount preparations were observed with a fluorescence microscope (Optiphot, Nikon) and photographed with a digital camera (Coolpix 990, Nikon).

Results

Intracellular and extracellular recordings from isolated opisthosomal ganglia

Each opisthosomal ganglion contains a pair of segmental nerves (dorsal and ventral nerves) with the dorsal nerves lying anterior to the ventral nerves. The dorsal nerves innervate visceral organs such as the intestine and the heart, and the ventral nerves innervate the muscles of the gill plates (Patten and Redenbaugh, 1900; Carlson, 1905). A cluster of cell bodies is located at the base of each segmental nerve.

Using intracellular electrodes, we located photosensitive neurons in single isolated opisthosomal ganglia. We also recorded extracellularly from dorsal or ventral nerves to correlate the activity of motor neurons with the activity of photosensitive neurons. Figure 1 illustrates the extracellular discharge pattern of the right ventral nerves of the 9th ganglion (Fig. 1A) and the intracellular response of a photosensitive neuron in the cluster of cell bodies of the same ganglion (Fig. 1B). In darkness, a periodic burst discharge, corresponding to gill plate activity, was recorded extracellularly from ventral nerves, and spontaneous action poten-

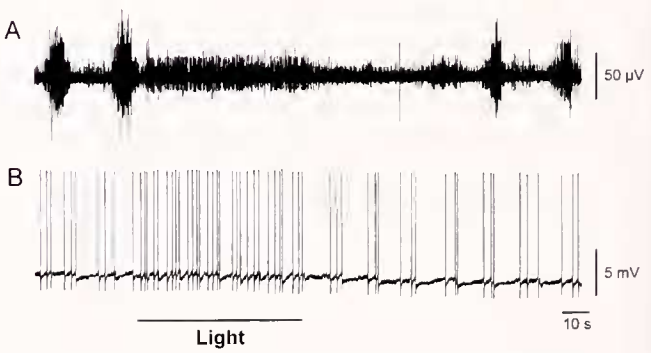

Figure 1. Simultaneous recordings of extracellular responses from the right ventral nerve in isolated 9th ganglion (A), and intracellular responses from a photosensitive neuron within a cluster of cell bodies in the same ganglion (B). In (A), illumination produced the inhibition of rhythmic burst activity and the appearance of sustained bursting. In (B), the frequency of action potentials increased by illumination.

tials with an irregular interval were recorded intracellularly from a single neuron. Illumination of the ganglion increased the frequency of action potentials and inhibited the periodic burst discharge of ventral nerves, producing a sustained discharge.

To determine whether the photosensitive neurons respond to light directly or through synapses, the preparation was treated with Co^{2+}, a blocker of chemical transmission. Figure 2 shows simultaneous recordings from the left ventral nerves of the 13th ganglion and a photosensitive neuron in the same ganglion before (A) and after (B) application of Co^{2+}. In the presence of 20 m*M* Co^{2+}, the periodic burst discharge was inhibited within 3 min, while the frequency of action potentials without illumination was not significantly changed. Illumination increased the frequency of action potentials (Fig. 2B), suggesting that this photosensitive neuron is a primary photoreceptor. The effects of Co^{2+} partially recovered after washout (not shown).

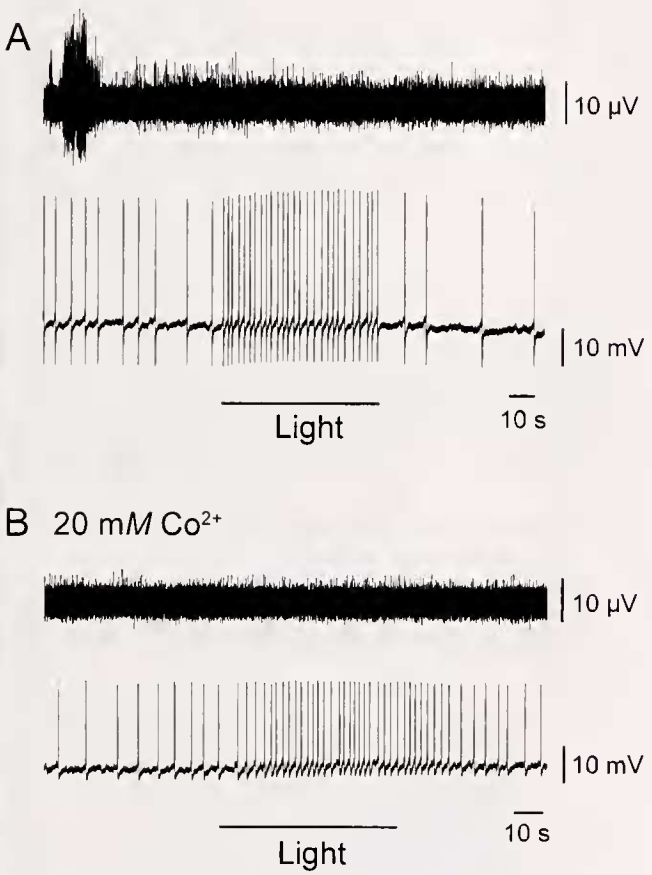

Figure 2. Effect of Co^{2+} on extracellular responses from the left ventral nerve in isolated 13th ganglion, and intracellular responses from a photosensitive neuron within a cluster of cell bodies in the same ganglion (A) before application of Co^{2+}; (B) 15 min after application of Co^{2+} (20 m*M*). Motor activity in the ventral nerve (upper trace) was reduced. The frequency of action potentials (lower trace) without illumination and the increase in the frequency of action potentials with illumination were not significantly changed in the presence of Co^{2+}.

Physiological properties of the photosensitive neurons

Photosensitive neurons are not abundant in isolated opisthosomal ganglia. Thus far, less than 2% of the neurons that we examined (20 out of 1050) exhibited photosensitivity. All 20 photosensitive neurons exhibited spontaneous action potentials in the dark and responded to light with an increase in the frequency of action potentials. The level of spontaneous activity and the response to light varied from cell to cell and, occasionally, during the course of experiment on the same cell. Three examples of photoresponses are shown in Figure 3. Responses just before and at the beginning of illumination (dotted line under each left trace) are shown on the right with an expanded time scale. Responses shown in Figure 3A and B were obtained from the same cell in the 12th ganglion. The cell had a resting potential of about −30 mV and exhibited few spontaneous action potentials in the dark within several minutes after penetration (Fig. 3A). In the course of the experiment, however, the frequency of action potentials gradually increased to about 1 impulse/s (ips) without a significant change in the membrane potential (Fig. 3B). After illumination, the membrane potential depolarized about 2 mV, and the frequency of action potentials increased to 2 ips. The latency of the response was 0.6 s in Figure 3A and 2 s in Figure 3B. The response in Figure 3C was recorded in the 15th ganglion. In the dark, the cell discharged an irregular spike frequency of about 0.1 ips. Light stimulation caused a slight depolarization of the membrane potential and increased the spike frequency to about 0.4 ips.

Figure 4 shows the effect of duration of light on a photosensitive neuron in the 12th ganglion. Light stimulation increased the frequency of action potentials from a rate of about 0.3 ips to 0.8 ips. The light-evoked response of 0.8 ips was independent of the stimulus duration up to 60 s, indicating minimal adaptation to the light stimulus. Photosensitive neurons did not exhibit substantial adaptation to light.

Photosensitive neurons have a graded response to light intensity. Figure 5 (A–F) shows responses recorded from a photosensitive neuron in the 9th ganglion to light of different intensities. The frequency of action potentials increased at light intensities greater than −2.0 log units, reaching a maximum of 0.9 ips at 0.0 log units. The number of action potentials to different light intensities was normalized to that evoked by the maximal light intensity (0.0 log units) and plotted against the log intensity of light for the three cells in Figure 5G. The data from the cell in Figure 5 (A–F) are illustrated by filled circles. The intensity-response curve indicates that the photosensitive neurons can convey information about light intensity over a 2-log unit.

Photosensitive neurons respond maximally to short wavelength illumination. Using different wavelengths of light, we examined the spectral response properties of five neurons. Figure 6 (A–D) shows a representative spectral response pattern of a photosensitive neuron in the 9th gan-

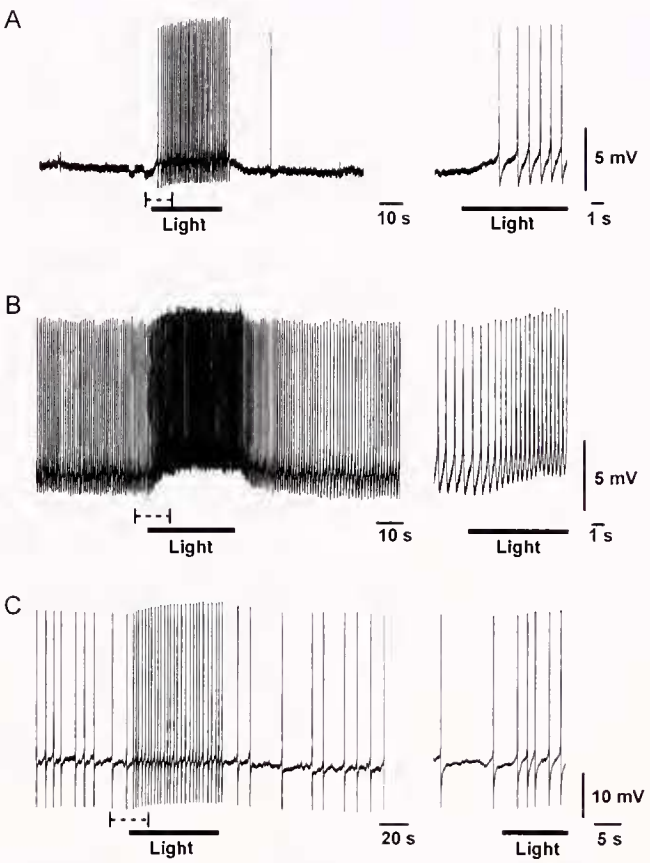

Figure 3. Three examples of the dark-adapted activity patterns and light responses of photosensitive neurons. (A and B) Responses from the same neuron in the cluster of cell bodies at the right dorsal nerve in an isolated 12th ganglion. In (A), the record was obtained 5 min after penetration. The cell was essentially quiescent in the dark. In (B), the record was obtained 80 min after the recording in (A). The frequency of action potentials in the dark increased without significant change in membrane potential. (C) Response from a neuron in the 15th ganglion. This neuron had a relatively low frequency of spontaneous action potentials with irregular spike intervals. All cells responded to light with an increase in the frequency of action potentials and a corresponding increase in the slope of the pacemaker potential, as seen in responses just before and at the beginning of illumination (dotted line under each left trace) and on the right with an expanded time scale.

glion. Monochromatic light of equal quantal flux but different wavelength was applied successively to the ganglion from 425 nm to 600 nm. The response increased slightly at 500 nm and became maximal at 425 nm. The number of action potentials at each wavelength was normalized with respect to the number of action potentials at 425 nm and plotted as a function of wavelength for five cells (Fig. 6E). The spectral-response curve suggests that photosensitive neurons are maximally sensitive at 425 nm or shorter wavelengths.

Morphological properties of the photosensitive neurons

All photosensitive neurons extend axons to the contralateral region of the ventral nerve cord, and most extend axons longitudinally in the cord. These morphological properties are based on observations of the Lucifer yellow (LY) that was iontophoretically injected into 12 photosensitive neurons after recording their electrical responses. Of 12 cells injected with LY, 6 were successfully stained. Figure 7 shows a photomicrograph of the cell in Figure 3A and B. The cell has an oval soma with a diameter of 103 μm (long axis) and no dendrites. A single axon extends toward the contralateral region without any branches, then turns, ascending in the connective toward the anterior ganglion (Figure 7, inset). Several thin branches are sparsely distributed along the axon within the 12th ganglion. Diagrams in Figure 8 show the 9th–16th opisthosomal ventral nerve cord with the recording sites of the 20 recorded photosensitive neurons marked by filled circles (Fig 8A) and 6 LY-stained photosensitive neurons (Fig. 8B). The diameter of the cell bodies ($n = 6$) was 102 ± 3 μm (long axis) and 75 ± 5 μm (short axis). Each cell extended its axon to the contralateral region. In five of the six cells, the axons ran along the longitudinal ventral nerve cord to either anterior or posterior connectives (Fig. 8B, circled A–D and F); the axon of the sixth cell was restricted in the ganglion (Fig. 8B, circled E).

Discussion

Extra-retinal photoreceptors that have been found in *Limulus* include ventral photoreceptors (Millecchia *et al.*,

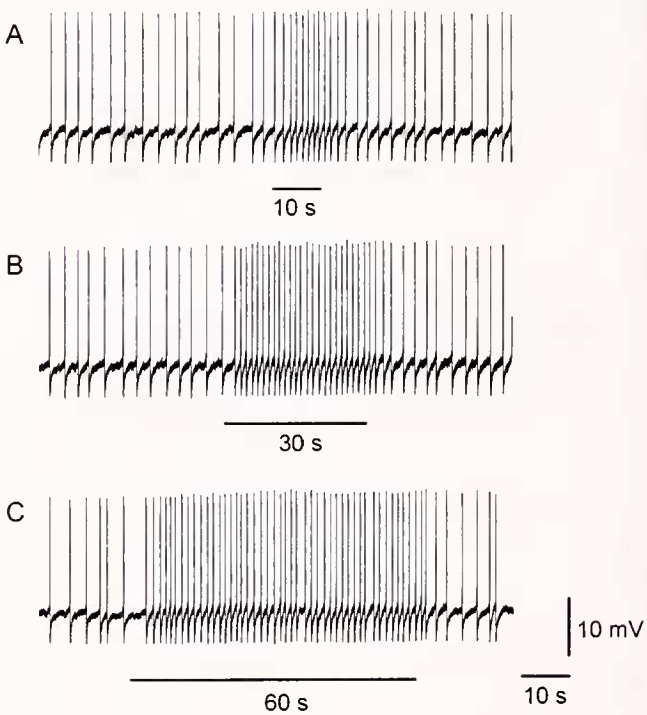

Figure 4. Effect of light of different duration on a photosensitive neuron within a cluster of cell bodies at the base of the right dorsal nerve of the 12th ganglion. An increase in the frequency of action potentials with illumination was maintained during illumination.

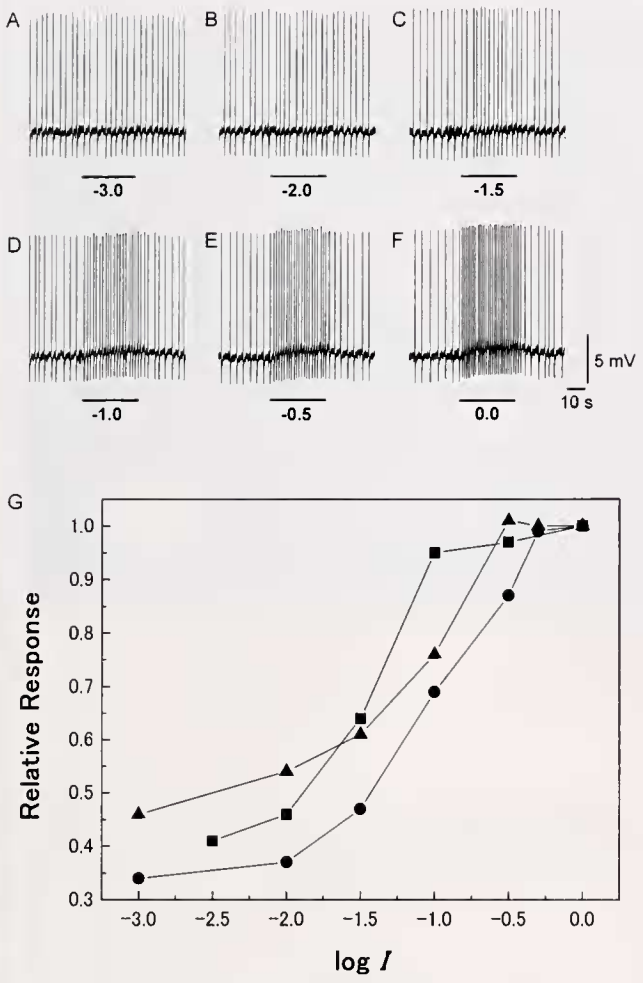

Figure 5. (A–F) Effect of light of different intensities on a photosensitive neuron within a cluster of cell bodies at the base of the left dorsal nerve of the 9th ganglion. Light stimuli between −3.0 and 0.0 log units were successively applied at intervals of several minutes. (G) The number of action potentials at each intensity of light was normalized with the number of action potentials at the maximum intensity of light and plotted against different intensities.

1966), photoreceptors adjacent to the optic ganglia (Snodderly, 1971), photoreceptors in the median optic nerves (Samie *et al.*, 1995), and photoreceptors in the telson (Hanna *et al.*, 1988). Here, we found new photosensitive neurons in the opisthosomal ventral nerve cord. These often exhibited spontaneous action potentials in the dark and an increase in the number of action potentials in light. The latency of the response to light was on the order of seconds. The frequency of action potentials was maintained during illumination and modulated by light intensity over a 2-log unit. The peak spectral response was at 425 nm or lower.

Are photosensitive neurons photoreceptors?

In *Limulus*, ventral photoreceptors and photoreceptor cells of the lateral and median eyes respond to light with a transient peak depolarization followed by a steady-state depolarization (Hartline *et al.*, 1952; Millecchia *et al.*, 1966; Nolte and Brown, 1972). On the other hand, the response of photosensitive neurons in the *Limulus* ventral nerve cord exhibits tonic discharges to light with little adaptation, which is similar to the response of the caudal crayfish photoreceptor (CPR) of the crayfish (Kennedy, 1958, 1963; Wilkens and Larimer, 1972). In the crayfish ventral nerve cord, primary photoreceptors are identified by at least two criteria: the production of a slow depolarization, with superimposed action potentials, upon illumination, and the

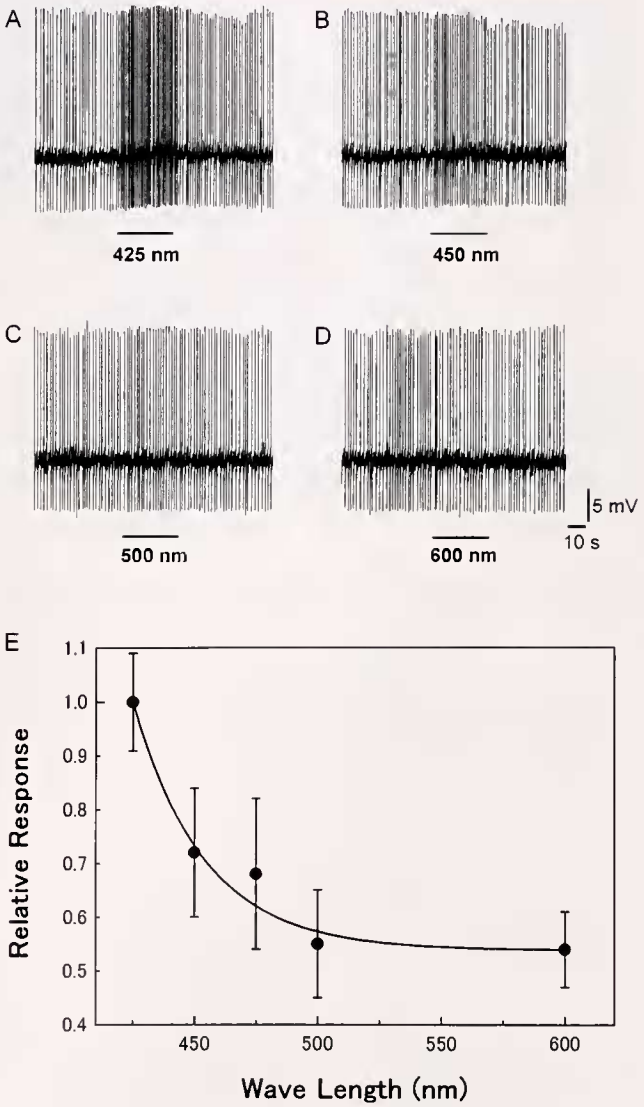

Figure 6. (A–D) Effect of light of different wavelengths on a photosensitive neuron within a cluster of cell bodies at the base of left dorsal nerve in the 9th ganglion. Monochromatic light with a duration of 30 s is shown below each record. Light stimuli were applied at intervals of several minutes. The maximum spectral response was seen at 425 nm. (E) The number of action potentials at each wavelength was normalized to the number of action potentials at 425 nm and plotted against different wavelengths. Values are mean (±SD).

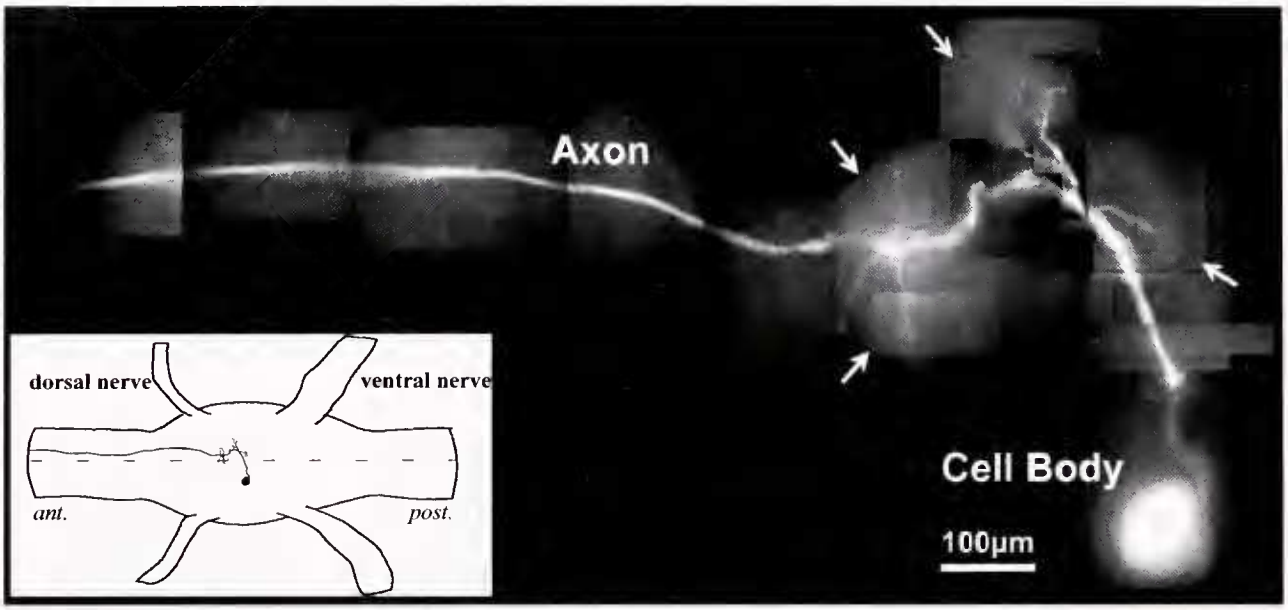

Figure 7. Ventral view of a Lucifer-yellow-injected photosensitive neuron in the 12th ganglion. The cell body (103 μm in diameter, long axis) extends an axon toward the contralateral region, where the axon turns sharply and ascends toward the 11th ganglion with some dendritic branches (arrows). Inset: a schematic drawing of the neuron in the ganglion.

resetting of the endogenous rhythm by interpolated antidromic impulses (Kennedy 1963). In the present study, many photosensitive neurons also responded to light with a slow depolarization and an increase in action potential frequency. Although we did not examine whether an antidromic pulse stimulation resets the spontaneous activity rhythm, the fact that, when chemical transmission was suppressed by Co^{2+}, photosensitive neurons did not lose spon-

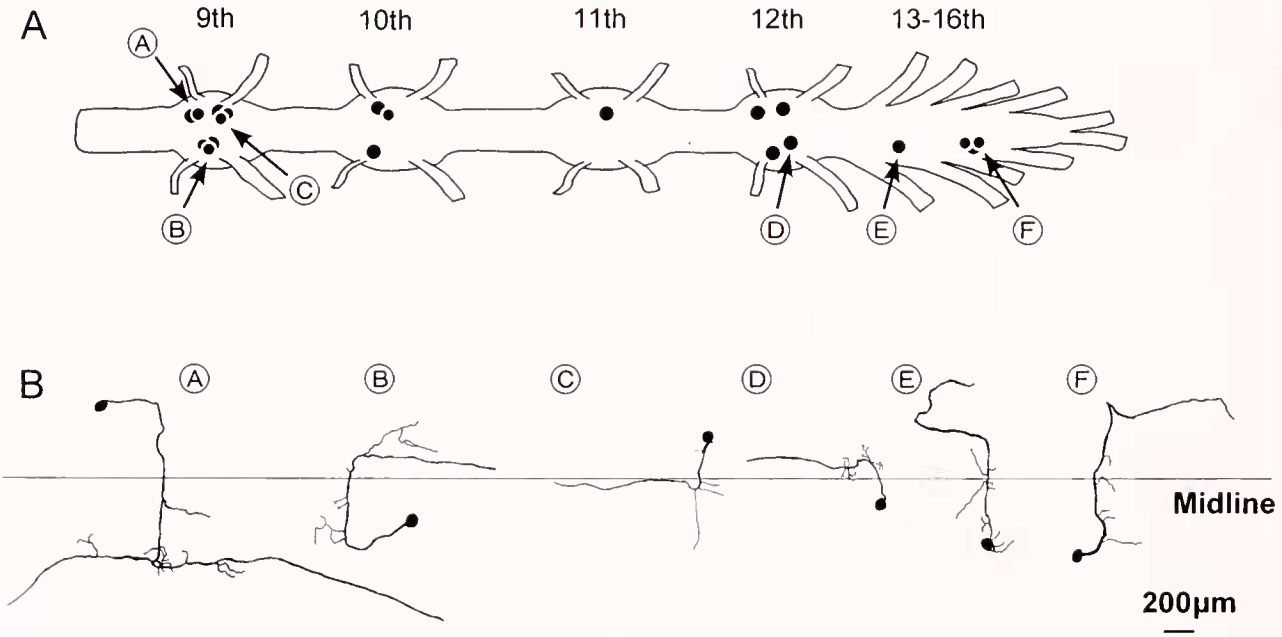

Figure 8. Ventral view of the opisthosomal ventral nerve cord (A), and the morphology of 6 Lucifer-yellow-labeled photosensitive neurons (B). In (A), filled circles indicate the location of somata of 20 photosensitive neurons that are examined physiologically. In (B), morphology of the labeled neurons is schematically illustrated. Circled letters in (B) correspond to those in (A). The midline of the opisthosomal ventral nerve cord is shown.

taneous action potentials in the dark and photosensitivity to light suggests that they are primary photoreceptors.

Comparison of extra-retinal photoreceptors in Limulus *and other arthropods*

From a phylogenetic point of view, it is important to point out similarities and differences in the extra-retinal photoreceptors of *Limulus* and other arthropods. Like *Limulus*, the scorpion is a chelicerate, and it shares a number of features in its structure and development with the horseshoe crab. It has been reported, using extracellular recording (Zwicky, 1968; Geethabali and Rao, 1973) and behavioral techniques (Zwicky, 1968, 1970), that the scorpion has extra-retinal photoreceptors in some abdominal ganglia, and that the peak spectral response is 568 nm with a secondary peak at 440 nm. A more detailed comparison between the extra-retinal photoreceptors of *Limulus* and the scorpion must await further analysis using intracellular recordings in scorpions. In the brain of spiders, extra-retinal photoreceptors with a peak spectral response at 420–440 nm have been detected using extracellular recording techniques (Yamashita and Tateda, 1981, 1983).

The photosensitive properties of *Limulus* neurons can be better compared to the caudal photoreceptor of the crayfish, which has been studied more extensively. The CPR in crayfish is composed of two bilaterally symmetrical neurons located in the 6th abdominal ganglion. Like those in *Limulus*, they exhibit spontaneous action potentials in the dark and a tonic increase in the frequency of action potentials in light (Prosser, 1934; Kennedy, 1963); but their peak spectral response is at 500 nm (Bruno and Kennedy, 1962; Larimer, 1966) rather than at 425 nm or lower. It is worth noting that the peak spectral response of extra-retinal photoreceptors in *Limulus* is similar to that in scorpion (the secondary peak) and spider, which are phylogenetically closer to the horseshoe crab.

The latency of the response in *Limulus* extra-retinal photoreceptors ranged from 0.5 to 5 s, with a mean ± SD value of 2.2 ± 1.1 s. Such a long latency has been also reported for CPR neurons (0.3–12 s, Kennedy, 1958). The large range of latencies may be partly due to measurement errors caused by a fluctuation in the intervals between spontaneous action potentials. Nevertheless, latencies on the order of seconds are significantly longer than those reported for lateral or ventral photoreceptors, which are on the order of milliseconds. A second messenger cascade has been suggested for the long response latency in crayfish CPR (Kruszewska and Larimer, 1993). The phototransduction mechanism remains unresolved for extra-retinal photosensitive neurons in *Limulus* ventral nerve cord.

Dye-injection experiments demonstrate that all somata of photosensitive neurons examined in *Limulus* extend an axon to the contralateral region, where it runs longitudinally along the opisthosomal ventral nerve cord in either or both directions. In crayfish, a pair of CPR interneurons also consists of a contralateral soma and ipsilateral axonal branches (Wilkens and Larimer, 1972). The axon of the CPR projects the length of the ventral nerve cord from the 6th ganglion to the brain (Simon and Edwards, 1990). In contrast, *Limulus* has photosensitive neurons in each ganglion of the ventral nerve cord. In one of six LY-stained cells, the axon was restricted to a single ganglion (Fig. 8B, circled E), whereas the other five cells extended their axons to anterior or posterior connectives (Fig. 8B, circled A–D and F). Therefore, it appears that they extend axons to more than one ganglion. Another morphological difference between the photoreceptor of the two species is the paucity of dendrites in *Limulus* relative to those in crayfish. Furthermore, in crayfish, these dendrites and not the cell body have been assumed to be photosensitive. The site of the light sensitivity of the photosensitive neurons in the opisthosomal ganglion of *Limulus* is not known.

Functional significance of extra-retinal photosensitive neurons

Extra-retinal photoreceptors in arthropods are often functionally associated with the entrainment of circadian rhythms (Yamashita and Tateda, 1981; Page, 1982). In crayfish, for example, circadian locomotor activity can be entrained by illumination of the brain (Page and Larimer, 1972). Furthermore, illumination of the CPR evokes leg movements (Welsh, 1934; Edwards, 1984; Simon and Edwards, 1990) and disrupts circadian locomotion (Fuentes-Pardo and Inclán-Rubio, 1987; Inclán-Rubio and Fuentes-Pardo, 1987). In scorpions, Zwicky (1968, 1970) has speculated that the negative phototaxis might be mediated by extra-retinal photoreceptors.

In the present and previous studies (Mori and Kuramoto, 2004), we have observed that illumination of isolated opisthosomal ganglia of the *Limulus* ventral nerve cord either inhibited or accelerated (or both) the activity of dorsal and ventral nerves. We suggest that light stimulation may modulate the activity of visceral organs and gill plates. In preliminary experiments, blue light from an LED embedded between the gill plates of a restrained animal accelerated the heart beat in some animals (data not shown). This result suggests that external light can reach the photosensitive neurons through the semitransparent skin of the intact animal and perhaps have a role in behaviors such as an animal righting itself. Photosensitive neurons may have an ever greater role in the behavior of juvenile horseshoe crabs because they are more transparent than adults. How environmental light modulates organ activities and animal behavior are subjects for future research.

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